STAIN TECHNOLOGY

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PROGRESS IN THE STANDARDIZATION OF STAINS

THE CERTIFICATION OF STAINS (*continued*)

In the last number of this journal (pp. 81–8) a summary was given of the work of the Stain Commission during the eleven years from 1923 to 1933 inclusive. At the end of this report it was stated that each of the dyes put on the certification basis had presented its own particular problems and that in the next number of the journal an attempt would be made to tell the story about some of these interesting problems. To do so for all the dyes that have been certified will require more space than is available in the present number of the journal. A beginning will be made, however, taking up some of the most interesting stains in alphabetical order, leaving it for future issues of the journal to complete the list.

BRILLIANT CRESYL BLUE

When the war shut off the supply of imported stains, brilliant cresyl blue was not available in America. This dye was no longer employed for any commercial purpose, as cheaper substitutes had been found for it. As a result, when pre-war stocks were exhausted domestic concerns either frankly told their customers they could not obtain it or tried to substitute some other dye of about the same shade. The best known use of the dye from the biological standpoint was as a blood stain to bring out reticulated corpuscles and platelets. Attempts were made to substitute crystal violet for these purposes, but demand for brilliant cresyl blue still continued.

This was one of the instances where stain companies were found extremely helpful. It was pointed out to us by the manufacturers that to supply biologists with this dye it would have to be prepared especially for them and would be so expensive to manufacture that there would be no hopes of getting back the money they would have to spend on it. Nevertheless, they gladly offered to do this and brilliant cresyl blue has been available on the American market ever since. There was never any question as to the quality of this dye, and as soon as its manufacture for biological purposes was begun the samples submitted have satisfied every test.

BRILLIANT GREEN

Brilliant green is still under investigation; samples submitted for certification are standardized at present only by the procedure of trial and error.

This dye has offered particular problems from the beginning, but not strictly as a biological stain. It is used almost exclusively for the sake of its inhibitory action on certain kinds of bacteria. Ordinarily the Stain Commission has not tested dyes or standardized them for such purposes. In the case of brilliant green, however, an exception was made quite early in the work because of the unusually large demand for it as a constituent of bacteriological media, especially of the brilliant green bile medium used in plating water in order to distinguish the colon organism from other lactose-fermenting bacteria. Tests for such purposes as this are time-consuming and often give results that are difficult to interpret. On this account it has not yet proved possible to correlate the toxicity of different samples of brilliant green with other characteristics of the samples. At the present time work along this line is in progress in co-operation with a referee of the Standard Methods Committee of the American Public Health Association. When the work is completed it is hoped that more will be learned as to why some lots are satisfactory and some are not. In the meantime all samples of brilliant green submitted for certification are tested to be sure they are satisfactory in the brilliant green bile medium. The standardization of this dye, therefore, is empirical rather than scientific.

CRESYL VIOLET

Cresyl violet is not yet standardized; there is so little demand for it, however, that it is not being especially investigated at present.

At the time of the war one of the German dyes for which there was little demand, but which nevertheless was particularly prized in certain special procedures because of its strong metachromatic properties, was usually known under the German name cresyl echt violet, a literal translation of which would be cresyl fast violet. This dye, like brilliant cresyl blue, was not used for textile dyeing. In fact, it had so little commercial value that it has never been listed in any of the dye indexes.

As a result of this fact, the exact chemical nature of imported cresyl violet is not definitely known. The present imported product seems to be essentially the same as that which was on the market before the war, but it is distinctly different from the American product now sold under the name of cresyl violet.

The American cresyl violet, manufactured by the National Aniline and Chemical Company, is closely related to Nile blue and has a definitely known chemical composition. Inasmuch as the dye is not extensively used, and the domestic product satisfies at least some of the purposes for which it is employed, no serious effort has been made to duplicate the German product. This is an instance where it is realized that satisfactory standardization has not been carried out, but it seems unwise to undertake the amount of research necessary until there is a greater demand for the dye than exists at the present time.

EOSIN ALCOHOL SOLUBLE (ETHYL EOSIN)

The problem of alcoholic eosin, originally quite puzzling, has now been completely solved.

Confusion in the labeling of eosin and its related dyes still exists among imported products; but at the outset of the work on standardization, it was found that mislabeling was very common also in the case of those of domestic manufacture—much more common than with any other group of dyes. Alcohol soluble eosin was a striking instance to the point, particularly because the name is somewhat misleading, ordinary eosin Y being readily soluble in both alcohol and water.

Nevertheless, even tho it were pointed out to a biologist that eosin Y was soluble in water, a brief test sufficed to show him that it was not what he wanted. He did not know what he needed, and as the stain companies were equally in the dark, they began experimenting by offering other products to the trade. One of these was the color acid of eosin Y. It is not incorrect to call this alcohol soluble eosin, as it is soluble in that reagent and about as insoluble in water as an organic product can be. Unfortunately, however, it is not a dye, and does not become such until converted into one of its water soluble salts, when it is no different from ordinary eosin Y. Naturally, such a product did not suit the microscopists.

The demand for alcohol soluble eosin had been reaching the Stain Commission for several years before it was finally learned what was really wanted. Then it was discovered that the dye sold under this name in Germany was the ethyl ester of eosin. As soon as the stain companies received this information they were readily able to manufacture it and apparently have been meeting the demands of biologists in this particular ever since in an entirely satisfactory way.

—H. J. Conn

(*To be continued*)

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stain approved since the last one listed in the July number of this journal.

STAINS CERTIFIED JUNE 1, 1934, TO AUGUST 31, 1934*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Brilliant cresyl blue	NV 13	64%	As vital blood stain	June 7, 1934
Safranin O	CS 6	91%	As bacteriological stain	June 21, 1934
Brilliant green	DBg 1	93%	As constituent of bacteriological media	July 7, 1934
Brilliant green	CBg 5	95%	As constituent of bacteriological media	July 7, 1934

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

THE TITRATION OF DYES FOR THEIR BACTERIOSTATIC ACTION[1]

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ABSTRACT.—**A study was made of the extrinsic bacteriostatic value of different lots of two brands of several tri-phenyl methane dyes. *Staphylococcus aureus* Rosenbach and *Bacterium communior* Holland were chosen as the test organisms. The conclusions are as follows: (1) Recent lots of the two brands of each dye possess practically the same bacteriostatic value; (2) the ethyl group in brilliant green appears to be responsible for the marked inhibition of the colon organism by this dye; (3) the bacteriostatic action of a dye is influenced by the H-ion concentration and constituents of the medium and by the amount of the inoculum; (4) the therapeutic value of a dye cannot be judged by tests in vitro.**

The Biological Stain Commission does not include bacteriostasis standards in its requirements for the certification of dyes. According to Conn (1930), bacteriostasis tests for the inhibition of the growth of bacteria are not included in these standards for two reasons: (1) because of insufficient assistance to make the necessary tests; (2) because of the possible false impression it may convey that a dye satisfactory for bacteriostatic purposes may be employed therapeutically. Conn (1930), however, states that the bacteriostatic effects of a dye may prove as valuable as the staining properties and he urges research in this field.

The purpose of this investigation was to compare the bacteriostatic action of crystal violet, gentian violet, methyl violet, brilliant green and malachite green upon a Gram-positive and a Gram-negative organism. The values of different lots and brands of the dyes were also compared.

MATERIALS AND METHODS

Sixteen samples of dyes were tested. Six samples were furnished thru the courtesy of the Coleman and Bell Company; two were old samples from the same company in the laboratory supply, one of crystal violet and one of brilliant green. The remaining eight samples

[1]This paper covers in part the work submitted to the Faculty of Smith College by the senior author in partial fulfillment of the requirements for the degree of Master of Arts.

were furnished thru the courtesy of the National Aniline and Chemical Company.

The test organisms were the orange pyogenic coccus, *Staphylococcus aureus* Rosenbach, and the colon organism, *Bacterium communior* Holland. The culture of *Staph. aureus* was a strain from the Smith College collection. It had a deep yellow-orange pigment. The culture of *Bact. communior* was from the same collection but had originally been obtained from the American Type Culture collection.

Preliminary tests revealed that this strain of *Staph. aureus* grew very slowly on agar containing 0.5% peptone and 0.3% beef extract which are the percentages recommended for the standard analysis of milk and water. The proportions recommended by Reddish (Kolmer and Boerner, 1931, p. 424) were tried. These amounts, namely 1.0% peptone and 0.5% beef extract, encouraged the growth of *Staph. aureus* and were used in the following experiments. Sodium chloride was omitted, however, in order to eliminate any possible effect it might have on the dye. The H-ion concentration of the media was adjusted to 6.8.

Since there are no specific standard methods for bacteriostasis tests, the methods employed were a modification of the Rideal-Walker, Hygienic Laboratory, and Reddish methods for testing disinfectants (Kolmer & Boerner, 1931, pp. 418–427). Altho these methods are very artificial, they seemed satisfactory for the purpose of comparing the bacteriostatic properties of similar chemicals.

Before testing, the cultures were transferred to plain nutrient broth at daily intervals for three or more days. The *Staph. aureus* culture was also transferred in the same way to agar slants since its growth in broth in 24 hours was very scant. The inoculum from the agar culture was much heavier than that from the broth culture, thus serving to indicate the influence of the amount of the inoculum on the titer.

The extrinsic method was used, that is, the dye was added to the medium. Dilutions were made aseptically in sterile water from a saturated alcoholic solution of each dye. The saturated alcoholic solutions were prepared by the filtration of supersaturated alcoholic solutions. The agar was tubed in 9 cc. amounts and sterilized. The dilutions of the dyes were computed so that 1 cc. of the aqueous dilution gave the desired concentration when added to 9 cc. of sterile agar.

A preliminary range-finding test was performed and concentrations selected from this range were tested with the organisms. Three to five checks were made on the concentrations in the range of the highest dilution which caused complete inhibition and in the range of the dilutions which allowed moderate or very slight growth in 48 hours.

TABLE 1. TITRATION WITH *Staph. aureus* (HEAVY INOCULUM)

Transfers from a 24 hour *agar* culture

Dilutions from a saturated alcoholic solution. Incubation temperature, 30° C.

Co.	Dye	Complete inhibition			Partial inhibition			No inhibition		
		48 hr.	4 da.	1 wk.	48 hr.	4 da.	1 wk.	48 hr.	4 da.	1 wk.
	Tri-amino tri-phenyl methane group									
CB	Crystal violet, lot No. 787	2,000	2,000	2,000	8,000	8,000	8,000	100,000	100,000	100,000
CB	Crystal violet, 84% C. I. No. 681, Cert. No. CC-5	25,000	20,000	8,000	35,000	35,000	25,000	1,000,000	1,000,000	1,000,000
CB	Methyl violet 6B	10,000	10,000	10,000	35,000	35,000	20,000	1,000,000	1,000,000	1,000,000
CB	Gentian violet, improved C. I. No. 680, Cert. No. CB-9	20,000	10,000	10,000	40,000	20,000	20,000	1,000,000	1,000,000	1,000,000
NAC	Methyl violet B, lot No. 6581	10,000	10,000	8,000	40,000	30,000	20,000	1,000,000	1,000,000	1,000,000
NAC	Methyl violet B, lot No. 4390	8,000	6,000	6,000	40,000	40,000	35,000	1,000,000	1,000,000	1,000,000
NAC	Crystal violet, lot No. 5924	20,000	8,000	8,000	40,000	40,000	25,000	1,000,000	1,000,000	1,000,000
NAC	Crystal violet, lot No. 6282	10,000	10,000	10,000	80,000	80,000	40,000	1,000,000	1,000,000	1,000,000
NAC	Gentian violet, medicinal lot No. 5919	10,000	10,000	10,000	20,000	20,000	15,000	1,000,000	1,000,000	100,000
NAC	Gentian violet, medicinal lot No. 6580	10,000	6,000	6,000	25,000	20,000	15,000	1,000,000	1,000,000	100,000
	Di-amino tri-phenyl methane group									
NAC	Brilliant green, medicinal lot No. 5841	50	50	50	100	100	100	100,000	100,000	100,000
NAC	Brilliant green, medicinal lot No. 6684	50	50	50	100	100	100	100,000	100,000	100,000
CB	Brilliant green	100	100	50	250	250	250	100,000	100,000	100,000
CB	Brilliant green, Cert. No. CBg-4	50	50	50	100	100	100	100,000	100,000	100,000
CB	Malachite green, 81% Cert. No. CMg-1	500	500	500	5,000	1,000	1,000	100,000	100,000	10,000
CB	Malachite green, 76% Cert. No. CMg-2	500	500	500	5,000	1,000	1,000	100,000	100,000	10,000

CB indicates Coleman and Bell Company; NAC indicates National Aniline and Chemical Company.

The *Bact. communior* cultures were incubated at 37° C. and the *Staph. aureus* cultures were incubated at 30° C. for one week. These were the optimum incubation temperatures for each organism.

Observations were made after 48 hours and also after four and seven days or more to detect any colonies produced by resistant cells.

Results

As shown by Tables 1 and 2, it required approximately four to ten times the strength of crystal violet, one hundred and fifty times the strength of malachite green and one thousand times the strength of brilliant green to inhibit completely a heavy inoculum from the agar slant culture of *Staph. aureus* as to inhibit a light inoculum from the broth culture. This shows the dependence of the bacteriostatic titer of a dye upon the amount of the inoculation.

Table 4 summarizes Tables 1, 2 and 3 and gives the bacteriostatic range of the dyes after a 48-hour incubation period. This range begins with the highest dilution which completely inhibited growth, according to the macroscopic appearance of the dye-agar slants, and ends with the lowest dilution which had no inhibiting effect on the growth. Table 4 also facilitates the ranking of the 16 samples of dyes in the order of their bacteriostatic values. (See Table 5.)

In order to compare the bacteriostatic titers of the dyes with a Gram-positive organism and with a Gram-negative organism, it is necessary that the conditions be the same in both cases. For this reason the titers of the dyes for the inoculum from the broth culture of *Staph. aureus* are omitted in Table 5, since it was considered that the inoculum from the agar slant culture of *Staph. aureus* and that from the broth culture of *Bact. communior* were nearly equal in amount. Table 5 gives the bacteriostatic titers for complete inhibition of the growth of the organism for an incubation period of 48 hours.

All of the tri-amino tri-phenyl methane dyes exhibited about the same titer for *Staph. aureus* except the old uncertified sample of crystal violet (Lot No. 787), which was decidedly lower. The di-amino tri-phenyl methane dyes inhibited *Staph. aureus* in very strong concentrations. The samples of malachite green and the old uncertified sample of brilliant green were slightly more bacteriostatic for the Gram-positive organism than the other dyes of this group.

The order of the dyes was not exactly reversed for *Bact. communior*. Unexpectedly the old sample of brilliant green had the highest inhibiting titer. The inhibiting strength of the medicinal and the other more recent samples of brilliant green was one-half that of the old sample of brilliant green. The titers of the two samples of malachite

TABLE 2. TITRATION WITH *Staph. aureus* (LIGHT INOCULUM)

Transfers from a 24 hour *broth* culture

Dilutions from a saturated alcoholic solution. Incubation temperature, 30° C.

Co.	Dye	Complete inhibition			Partial inhibition			No inhibition		
		48 hr.	4 da.	1 wk.	48 hr.	4 da.	1 wk.	48 hr.	4 da.	1 wk.
	Tri-amino tri-phenyl methane group									
CB	Crystal violet, lot No. 787	8,000	6,000	6,000	10,000	10,000	10,000	1,000,000	100,000	100,000
CB	Crystal violet, 84% C. I. No. 681, Cert. No. CC-5	80,000	80,000	40,000	100,000	80,000	80,000	1,000,000	1,000,000	1,000,000
CB	Methyl violet 6B	80,000	80,000	40,000	100,000	100,000	80,000	1,000,000	1,000,000	1,000,000
CB	Gentian violet, improved C. I. No. 680, Cert. No. CB-9	80,000	80,000	40,000	100,000	100,000	80,000	1,000,000	1,000,000	1,000,000
NAC	Methyl violet B, lot No. 6581	80,000	40,000	40,000	100,000	100,000	100,000	1,000,000	1,000,000	1,000,000
NAC	Methyl violet B, lot No. 4390	80,000	40,000	40,000	100,000	100,000	100,000	1,000,000	1,000,000	1,000,000
NAC	Crystal violet, lot No. 5924	80,000	40,000	40,000	100,000	100,000	100,000	1,000,000	1,000,000	1,000,000
NAC	Crystal violet, lot No. 6282	80,000	40,000	40,000	100,000	100,000	100,000	1,000,000	1,000,000	1,000,000
NAC	Gentian violet, medicinal lot No. 5919	100,000	80,000	80,000	Above 100,000	100,000	100,000	2,000,000	1,000,000	1,000,000
NAC	Gentian violet, medicinal lot No. 6580	100,000	80,000	80,000	Above 100,000	100,000	100,000	2,000,000	1,000,000	1,000,000
	Di-amino tri-phenyl methane group									
NAC	Brilliant green, medicinal lot No. 5841	80,000	80,000	80,000	1,000,000	1,000,000	1,000,000	Above 1,000,000	Above 1,000,000	1,000,000
NAC	Brilliant green, medicinal lot No. 6684	80,000	80,000	40,000	1,000,000	1,000,000	100,000	Above 1,000,000	Above 1,000,000	1,000,000
CB	Brilliant green (old sample)	80,000	80,000	80,000	Above 1,000,000	Above 1,000,000	1,000,000	Above 1,000,000	Above 1,000,000	Above 1,000,000
CB	Brilliant green 90% Cert. No. CBg-4	80,000	80,000	80,000	Above 1,000,000	Above 1,000,000	1,000,000	Above 1,000,000	Above 1,000,000	Above 1,000,000
CB	Malachite green 81% Cert. No. CMg-1	80,000	40,000	40,000	1,000,000	100,000	100,000	Above 1,000,000	1,000,000	1,000,000
CB	Malachite green 76% Cert. No. CMg-2	80,000	40,000	40,000	Above 100,000	100,000	100,000	1,000,000	1,000,000	1,000,000

CB indicates Coleman and Bell Company; NAC indicates National Aniline and Chemical Company.

TABLE 3. TITRATION WITH *Bact. communior*

Transfers from a 24 hour *broth* culture

Dilutions from a saturated alcoholic solution. Incubation temperature, 37° C.

Co.	Dye	Complete inhibition			Partial inhibition			No inhibition		
		48 hr.	4 da.	1 wk.	48 hr.	4 da.	1 wk.	48 hr.	4 da.	1 wk.
	Tri-amino tri-phenyl methane group									
CB	Crystal violet, lot No. 787	100	90	Below 90	200	200	100	10,000	10,000	1,000
CB	Crystal violet 84% C. I. No. 681, Cert. No. CC-5	200	100	100	300	200	200	10,000	10,000	10,000
CB	Methyl violet 6B	300	200	200	400	400	400	10,000	10,000	10,000
CB	Gentian violet, improved C. I. No. 680, Cert. No. CB-9	200	200	200	400	400	300	Above 10,000	10,000	10,000
NAC	Methyl violet B, lot No. 6581	200	200	200	750	400	300	10,000	10,000	10,000
NAC	Methyl violet B, lot No. 4390	200	200	200	750	400	300	10,000	10,000	10,000
NAC	Crystal violet, lot No. 5924	200	200	100	400	200	200	10,000	10,000	10,000
NAC	Crystal violet, lot No. 6282	100	100	100	500	300	300	10,000	10,000	10,000
NAC	Gentian violet, medicinal lot No. 5919	75	75	75	200	200	200	10,000	10,000	10,000
NAC	Gentian violet, medicinal lot No. 6580	75	75	75	100	100	100	10,000	10,000	10,000
	Di-amino tri-phenyl methane group									
NAC	Brilliant green, medicinal lot No. 5841	10,000	6,000	5,000	20,000	15,000	15,000	100,000	40,000	40,000
NAC	Brilliant green, medicinal lot No. 6684	10,000	5,000	2,500	15,000	7,500	5,000	100,000	40,000	25,000
CB	Brilliant green (old sample)	20,000	7,500	Below 5,000	35,000	20,000	5,000	Above 100,000	100,000	100,000
CB	Brilliant green 90% Cert. No. CBg-4	10,000	5,000	Below 5,000	15,000	10,000	5,000	100,000	40,000	20,000
CB	Malachite green 81% Cert. No. CMg-1	2,000	2,000	2,000	4,000	4,000	2,000	8,000	6,000	4,000
CB	Malachite green 76% Cert. No. CMg-2	2,000	1,000	1,000	4,000	4,000	2,000	8,000	6,000	6,000

CB indicates Coleman and Bell Company; NAC indicates National Aniline and Chemical Company.

green were five times lower than the medicinal and certified samples of brilliant green. The tri-amino tri-phenyl methane group showed very low titers for the complete inhibition of *Bact. communior*. Crystal violet, methyl violet and gentian violet inhibited *Bact. communior* in concentrations of the same strength as were required by

TABLE 4. BACTERIOSTATIC RANGE FROM HIGHEST DILUTION GIVING COMPLETE INHIBITION TO LOWEST NON-INHIBITING DILUTION AFTER AN INCUBATION PERIOD OF 48 HOURS

Co.	Dye	*Staph. aureus* Heavy inoculum		*Staph. aureus* Light inoculum		*Bact. communior*	
		from	to	from	to	from	to
CB	Crystal violet, lot No. 787 (old sample)	2,000	100,000	8,000	1,000,000	100	10,000
CB	Crystal violet 84% C. I. No. 681, Cert. No. CC-5	25,000	1,000,000	80,000	1,000,000	200	10,000
CB	Methyl violet 6B	10,000	1,000,000	80,000	1,000,000	300	10,000
CB	Gentian violet, improved C. I. 680, Cert. No. CB-9	20,000	1,000,000	80,000	1,000,000	200	10,000
NAC	Methyl violet B, lot No. 6581	10,000	1,000,000	80,000	1,000,000	200	10,000
NAC	Methyl violet B, lot No. 4390	8,000	1,000,000	80,000	1,000,000	200	10,000
NAC	Crystal violet, lot No. 5924	20,000	1,000,000	80,000	1,000,000	200	10,000
NAC	Crystal violet, lot No. 6282	10,000	1,000,000	80,000	1,000,000	100	10,000
NAC	Gentian violet, medicinal lot No. 5919	10,000	1,000,000	100,000	2,000,000	75	10,000
NAC	Gentian violet, medicinal lot No. 6580	10,000	1,000,000	100,000	2,000,000	75	10,000
NAC	Brilliant green, medicinal lot No. 5841	50	100,000	80,000	Above 1,000,000	10,000	100,000
NAC	Brilliant green, medicinal lot No. 6684	50	100,000	80,000	Above 1,000,000	10,000	100,000
CB	Brilliant green (old sample)	100	100,000	80,000	Above 1,000,000	20,000	Above 100,000
CB	Brilliant green 90% Cert. No. CBg-4	50	100,000	80,000	Above 1,000,000	10,000	100,000
CB	Malachite green 81% Cert. No. CMg-1	500	100,000	80,000	Above 1,000,000	2,000	8,000
CB	Malachite green 76% Cert. No. CMg-2	500	100,000	80,000	1,000,000	2,000	8,000

the di-amino group to inhibit *Staph. aureus*. The medicinal samples of gentian violet showed the least inhibitive action on *Bact. communior*.

Referring again to Table 5, different lots and brands of the same dye revealed little variation in their bacteriostatic effects. Methyl violet B (Lot No. 6581) was slightly more inhibitive for *Staph. aureus* than was methyl violet B (Lot No. 4390). This was the only dis-

crepancy in different lots of the same dye from the same manufacturer except for the old samples of brilliant green and of crystal violet.

Readings recorded after four and seven days incubation showed a lowering of the titers on account of the growth of resistant cells. The dyes, however, remained in the same order with less distinction between those of almost equal value. (See Tables 1, 2 and 3.)

TABLE 5. BACTERIOSTATIC VALUES—TITERS FOR COMPLETE INHIBITION IN 48 HOURS

Staph. aureus			*Bact. communior*		
Co.	Dye	Titer	Co.	Dye	Titer
CB	Crystal violet 84% C. I. No. 681, Cert. No. CC-5	1:25,000	CB	Brilliant green (old sample)	1:20,000
CB	Gentian violet, improved C. I. No. 680, Cert. No. CB-9	1:20,000	NAC	Brilliant green, medicinal lot No. 5841	1:10,000
NAC	Methyl violet B, lot No. 6581	1:10,000	NAC	Brilliant green, medicinal lot No. 6684	1:10,000
CB	Methyl violet 6B	1:10,000	CB	Brilliant green 90% Cert. No. CBg-4	1:10,000
NAC	Crystal violet, lot No. 5924	1:10,000	CB	Malachite green 81% Cert. No. CMg-1	1:2,000
NAC	Crystal violet, lot No. 6282	1:10,000	CB	Malachite green 76% Cert. No. CMg-2	1:2,000
NAC	Gentian violet, medicinal lot No. 5919	1:10,000	CB	Methyl violet 6B	1:300
NAC	Gentian violet, medicinal lot No. 6580	1:10,000	CB	Crystal violet 84% C. I. No. 681, Cert. No. CC-5	1:200
NAC	Methyl violet B, lot No. 4390	1:8,000	CB	Gentian violet, improved C. I. No. 680, Cert. No. CB-9	1:200
CB	Crystal violet, lot No. 787	1:2,000	NAC	Methyl violet B, lot No. 6581	1:200
CB	Malachite green 81% Cert. No. CMg-1	1:500	NAC	Methyl violet B, lot No. 4390	1:200
CB	Malachite green 76% Cert. No. CMg-2	1:500	NAC	Crystal violet, lot No. 5924	1:200
CB	Brilliant green (old sample)	1:100	NAC	Crystal violet, lot No. 6282	1:100
NAC	Brilliant green, medicinal lot No. 5841	1:50	CB	Crystal violet, lot No. 787	1:100
NAC	Brilliant green, medicinal lot No. 6684	1:50	NAC	Gentian violet, medicinal lot No. 5919	1:75
CB	Brilliant green 90% Cert. No. CBg-4	1:50	NAC	Gentian violet, medicinal lot No. 6580	1:75

The violet dyes possessed a wide bacteriostatic range for *Staph. aureus* but complete inhibition ended at a high dilution, 1:10,000 or above. This same dilution had no effect on the growth of *Bact. communior* since the inhibiting range for *Staph. aureus* was definitely above that for *Bact. communior*. (See Table 4.)

Brilliant green also showed a wide bacteriostatic range for *Staph. aureus* but it was lower than that of the tri-amino group. It completely inhibited the Gram-positive organism up to a dilution of 1:100

and at a dilution of 1:100,000 it caused no inhibition. *Bact. communior*, however, was inhibited within a narrower range. Complete inhibition ended at a comparatively high dilution, 1:10,000; also no inhibition began at a dilution of 1:100,000. Consequently, a concentration of brilliant green that completely inhibited *Bact. communior* also had a slight inhibitory effect on *Staph. aureus*.

Malachite green inhibited *Bact. communior* within a very limited and low range of dilutions, complete inhibition ending at a dilution of 1:2000 and no inhibition beginning at a dilution of 1:8,000. This narrow range for *Bact. communior* was overlapped by a moderately wide range for *Staph. aureus*. As in the case of brilliant green, a concentration of malachite green that completely inhibited *Bact. communior* was also slightly bacteriostatic for *Staph. aureus*. However, a concentration such as 1:8,000, which had no effect on the growth of *Bact. communior*, was still somewhat bacteriostatic for *Staph. aureus*.

Growth on the dye-agar slants began at the bottom of the slant because of the water of syneresis, which Hadley (1926, p. 179) says aids dissociation; but it also dilutes the dye.

Effect of the growth of the organisms on the dyes. The dye-agar media were decolorized by the organisms. Decolorization was not perceptible in the very strong concentrations of crystal violet such as 1:100 to 1:500. Knowing from previous experiments that decolorization of the dye was due to alkalinization, H-ion concentration tests were performed daily for seven successive days to compare the interrelationship of alkalinization, growth, involution forms and decolorization of the dye-agar with that of the plain agar control.

Alkalinization of the dye-agar proceeded at the same rate as that of the plain agar altho growth was slightly retarded. In the presence of the dye, atypical forms, such as filaments, were abundant in *Bact. communior* cultures in the vicinity of the neutral point. The filaments disappeared as the alkalinity of the medium increased and the cells were normal in morphology when the pH of the medium reached 8.0. At a pH of 8.0, the dye in the medium was completely decolorized.

Discussion

Crystal violet, a tri-amino tri-phenyl methane dye, is especially toxic for the Gram-positive group of organisms; but dyes with one less amino group, (brilliant green, malachite green) are more toxic for the Gram-negative group of organisms. The presence, however, of the sulfate radical and of ethyl groups instead of methyl groups in a di-amino tri-phenyl methane dye, seems to make brilliant green es-

pecially toxic for *Bact. communior* and perhaps for other Gram-negative organisms. The fact that the chemical structure of malachite green resembles crystal violet, in having its hydrogen atoms substituted by the methyl group, and resembles brilliant green, in being a di-amino dye, suggests that its bacteriostatic titer would be between those of the other two dyes. Such was the case in this investigation.

The general order of the dyes given in Table 5 would doubtless remain the same for other organisms. Poe (1930) found this to be so when anaerobes were used as the test organisms. Of course, the titer of each dye would vary with the species and strain; therefore, a dye can be used to separate closely related strains (Churchman, 1912). The titers obtained in this experiment do not coincide with those given by Rakieten and Rettger (1927) for brilliant green and the colon organism, nor with those given by Stovall (1928) for crystal violet and the colon organism; but in each case certain conditions, such as culture media, methods and strains, differed from those stated in this report.

The titer is dependent on the amount of the inoculum, the composition of the medium, the pH of the medium, and the incubation temperature. Table 4 shows the influence of different amounts of the inoculum. Churchman (1921) stated that if the effect upon a single cell was known, the effect upon a group of cells would not be divisible by the number of cells on account of the communal activity of bacteria, i. e., the work done by a number of bacteria is more than an exact multiple of that done by one organism.

According to Kligler (1918), as long as the percentage of peptone and beef extract in the medium has no effect on the organism, it also has no influence on the bacteriostatic power of the dye. In order to compare results on different organisms, it is important that each test organism be in its optimum medium.

Salt was not used in the culture media in these experiments since it was not known what effect it would have on the dye. The investigations by Winslow (1923) suggest that it might influence the viability of the colon organism.

The H-ion concentration of the medium is of utmost importance. Rakieten and Rettger (1927) demonstrated the necessity of controlling the pH of brilliant green enrichment medium by means of a buffer. An alkaline reaction, as a by-product of metabolism, destroys the inhibitive power of the dye; whereas Stearn (1933) found that an initial H-ion concentration on the alkaline side of the isoelectric point of the dye enhanced the bacteriostatic effect. Yuri (1930) also

claims that the affinity of a dye for the bacterial cell is controlled by the H-ion concentration. Ingraham (1933) bases the bacteriostatic value of gentian violet on the oxidation-reduction potential of the medium; therefore, H-ion concentration is the prime factor.

Concerning the incubation temperature it is only necessary to repeat that each culture should be incubated at its optimum temperature.

Heat enhances the toxicity of gentian violet for *Staph. aureus* (Shands, 1928) which is to be expected if the phenomenon is a chemical reaction and if the Gram reaction of *Staph. aureus* is changed by heat (Churchman, 1929).

Before any therapeutic value can be attributed to a dye, other factors must be considered. In the animal body the situation is quite complicated. Beckwith (1921) stated that the addition of bile or serum does not affect the bacteriostatic titer of gentian violet. On the other hand, the histiocytic system may consume the dye. According to Beckwith (1921), the particular tissues instrumental in removing the dye *in vivo* are specific for the dye.

Conclusions

1. The bacteriostatic value of each dye remains fairly constant for different lots of the same brand and for the two brands investigated.

2. Since malachite green contains methyl groups and brilliant green contains ethyl groups, one may conclude that the ethyl group is especially toxic for the colon organism and is responsible for its inhibition by brilliant green.

3. The bacteriostatic values of the dyes are easily influenced by the H-ion concentration and constituents of the medium and by the amount of the inoculum. Consequently, experimental data in bacteriostasis tests should be recorded in detail. Standard methods should be devised for evaluating the bacteriostatic power of a dye.

4. Tests which determine the bacteriostatic value of a dye *in vitro* do not determine its therapeutic value.

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MARCHI'S STAINING METHOD: II. FIXATION

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ABSTRACT.—**Following experimental lesions, spinal cords of cats and rabbits were fixed with acid, neutral, and alkaline solutions. Staining was limited to a chromate-osmic (Marchi's) solution and a chlorate-osmic solution. The following conclusions were drawn:**

The presence of an acid in the fixative caused normal myelin sheaths to stain. This effect was reduced by washing tissues before staining, by adding acetic acid to the stain, or by employing a non-formalin fixative. It was, however, at no time entirely obviated.

A study was made of the granular deposits which occur in nearly all Marchi preparations and which are especially confusing if very light backgrounds are obtained.

The staining reactions of the granular deposits were very similar to those of degenerating myelin but some suppression of the granules was obtained by adding $KClO_3$ to the formalin fixative.

In an earlier investigation of the principles underlying the staining of degenerating myelin with the Marchi stain,[2] formalin fixation followed by staining in Marchi's solution plus 2% acetic acid gave marked differential staining; the background was very light and the degeneration stained distinctly. Granular deposits resembling dust, however, were present in the interstitial or neuroglial spaces, which became more noticeable as the background was lightened. Similar granular deposits were encountered in regular Marchi preparations but the darker background tended to obscure the "dust". Recently, Duncan[3] stated that most standard Marchi preparations show granular deposits, the degree of which varies in different animals. Iwana,[4]

[1]Contribution No. 206.

[2]Swank, R. L., and Davenport, H. A. Marchi's staining method: Studies of some of the underlying mechanisms involved. Stain Techn., **9**, 11–19. 1934.

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Allen,[5] Fischer,[6] and Keher[7] have also made similar reports, and we believe that most investigators either fail to notice this defect on account of the density of their preparations or take it as a matter of course. It is a serious handicap if one is studying fine and scattered degeneration, hence an understanding of its staining reactions, with the object of eliminating it, is desirable. The following experimentation was undertaken to determine the effect which variations in fixation have upon the staining of central nervous system tissues, with special attention paid to the granular deposits.

Material and Methods

Both degenerating and normal spinal cords of the cat were used. As we were interested primarily in fixation, staining was limited to the use of: (1) a dichromate-osmic mixture (Marchi's stain), either with or without 2% acetic acid (Groups A, B, C, D, F, G, and H; Table 1); and (2) a chlorate-osmic mixture ($KClO_3$ .25% and osmic acid .25%—Group E only; Table 1). Fixation, on the other hand, was varied and is described in the table. Unless otherwise specified fixation was for 24 hours.

Results

The effect of acidification of the fixative upon staining. When an acid was present in the fixative we found that staining of normal myelin rings appeared (Fig. 3—monochloracetic acid 1% in formalin 10%, followed by staining in Marchi's stain). This condition varied with the acid used, being the least marked with acetic acid and most marked with trichloracetic acid, with intermediate variations occurring in the order indicated in the table (exps. 3 to 6). The addition of an oxidizing agent ($K_2Cr_2O_7$ or $KClO_3$) to an acidified fixative did not noticeably inhibit the staining of the normal myelin rings, as the staining seemed to be predetermined by the presence of an acid in the fixative (Group C). Furthermore, it was found that the strength of the acid added to the formalin determined the degree to which the normal myelin stained; a 1% monochloracetic acid produced a denser staining than 0.5%. Sufficient increase in the acid content of the fixative caused the neuroglia to stain also (exps. 7, 8,

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TABLE 1

Group	Exp. No.	Fixative	Staining of myelin rings	Neuroglia	Dust
A	1	Formalin 10%[8]	—[9]	—[9]	+++[10]
	2	Formalin 10% Acetate buffer[11]	±	—	++
	3	Formalin 10% Acetic acid 3%	+	—	+
	4	Formalin 10% Formic acid ½%	++	—	+
	5	Formalin 10% Monochloracetic ½%	+++	—	+
	6	Formalin 10% Trichloracetic ½%	++++	—	Indistinct
	7	Formalin 10% Picric acid (Sat.)	+++++	++++	Indistinct
B	8	Trichloracetic 5%	++++[12]	±	Indistinct
	9	Sulfosalicylic 5%	++[12]	++	Indistinct
	10	Picric acid (Sat.)	+++[12]	+++	Indistinct
C	11	Formalin 10% $K_2Cr_2O_7$ 3% Acetic acid 3%	+	—	++
	12	$K_2Cr_2O_7$ 3% Acetic Acid 2%	+++[12]	+	Indistinct
	13	Formalin 10% $KClO_3$ 1% Acetic acid 3%	+	+	+++
	14	Formalin 10% $KClO_3$ 1% Acetic acid 5%	+++	—	±
D	15	Formalin 10% $K_2Cr_2O_7$ 2½%	—	—	+++
	16	Formalin 10% NH_4OH 1%	—	—	++++
E	17	Formalin 10%	—	±	—
	18	Formalin 10% $KClO_3$ 1%	—	—	—
	19	Formalin 10%	±	—	—
	20	Formalin 10% $KClO_3$ 1% Acetic acid 5%	—	—	—
F	21	Fixation omitted	+	±	++
	22	Formalin 10% (3 hours)	±	±	+++
	23	Formalin 10% (6 hours)	—	—	+++
	24	Formalin 10% (24 hours)	+	+	+++
G	25	Formalin 10% $KClO_3$ 1% (48 hours)	—	—	++
	26	Formalin 10% $KClO_3$ 5% (48 hours)	—	—	++
H	27	Formalin 20% (48 hours)	±	±	++++

[8]Controls were run for every animal included in the study, but for simplicity only one control is included in the table.

[9]Refers to the depth and uniformity of staining.

[10]Refers to the abundance of dust-like particles.

[11]0.05 molar, pH 5.2.

[12]The density of the stained myelin rings was markedly decreased.

9, and 10), thus showing that acid can influence the staining of normal myelin sheaths and neuroglia.

Two pieces of tissue from the same animal, one which had been fixed for 24 hours, and the second for one year were stained in Marchi's solution plus 2% acetic acid. The first piece of tissue (fixed 24 hours), stained differentially, possessed a minimum of dust and a complete absence of stained normal myelin rings. The second (fixed one year) showed a moderate number of stained normal myelin rings, an abundance of granular deposits and a much darker background than the first. Compare Figs. 5 and 6. We had observed previously that fixation for one to two months caused a marked increase in granular deposits. Consequently, it appears that as the time of fixation is lengthened, the staining of the granular deposits increases. At the same time changes in the normal myelin take place, but much slower, so that the expiration of a greater period of time is necessary before it will stain.

Another piece of tissue fixed for 2 days in formalin was put into 5% acetic acid for one day before staining. A staining of normal myelin rings comparable to that occurring in the tissues fixed for one year resulted, but there was no increase in interstitial granular deposits. This suggests that the increased staining of granular deposits following fixation for 1 to 2 months, and the staining of normal myelin rings which occurs after one year of fixation is due to a gradual action of the formalin. Apparently the pH change is very slight and the tissues are altered slowly, as only a moderate acid effect resulted after one year's fixation. Also, 5% acetic acid treatment following formalin fixation produces only a moderate staining of normal myelin rings, whereas 5% acetic acid added to the initial fixative produces a very marked result.

Factors which alter the effect of acid upon staining. A. Washing tissues following acid formalin fixation reduced the staining of normal myelin rings and neuroglia.[13] This effect was similar to the crenation of degenerating globules which is caused by washing tissues in water following fixation. In an experiment in which the degenerating tissue was fixed in 10% formalin, washed, then returned into formalin before staining in Marchi's solution, crenation was present. This indicates that the solubility (or emulsification) of the degenerating myelin, as well as the removal of formalin from the tissues, is a probable factor in the production of shriveled globules of degenerating myelin. Normal myelin also appeared to be softened and distorted by water.

B. The addition of acetic acid to the stain[13] reduced the staining

[13]These experiments are not included in the table.

of normal myelin rings. Also, the employment of a chlorate-osmic stain reduced the staining of normal myelin rings (Group E).

C. Formalin when used in the fixative was found to increase the staining of all tissue elements. In experiments 8, 9, 10, and 12 the tissues were fixed in an acid solution without formalin. Even tho the normal myelin stained, these preparations were lighter than those in Group A in which formalin and acid were employed in the fixative.

Ammonium hydroxide in the fixative. Ammonium hydroxide had very little effect upon the staining (exp. 16). It seems to act in an opposite manner to the acid by preventing the normal myelin and neuroglia from staining.

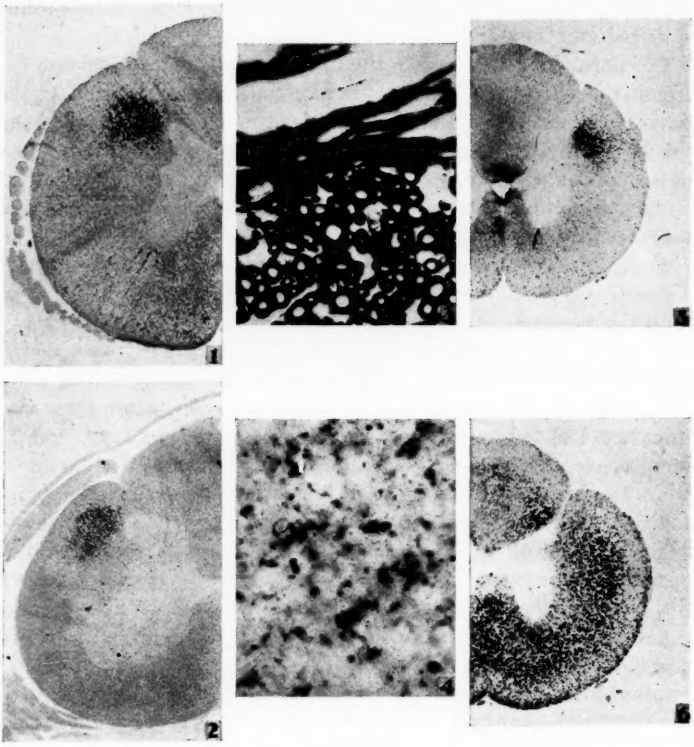
Variation in concentration of formalin and time of fixation. Twenty per cent formalin gave darker stains than 10% (exp. 27).

Twenty-four hours seems to be a safe minimum time for formalin fixation of small pieces of central nervous system (brain stem and spinal cord of cats and rabbits). Experiments 21, 22, 23, and 24 show the results of no fixation prior to staining and of 3, 6, and 24 hours fixation in formalin. It is evident that 6 hours' fixation is insufficient, while 24 hours is adequate. For larger pieces of tissue in which penetration to the center of the block would require a much greater time, a longer period for fixation would be necessary. Even the smallest piece of tissue seems to require about 24 hours; hence penetration time alone is not sufficient.

Addition of an oxidizing agent to formalin. Little or no change resulted from the addition of $K_2Cr_2O_7$ to formalin (exp. 15). This oxidizing agent exerted a very weak action, if any, upon the tissue, as was previously shown. Also the small amount of dichromate carried into the stain by the tissue caused no noticeable change, while doubling the amount of $K_2Cr_2O_7$ in the stain produced very little if any alteration in the staining.

The addition, however, of $KClO_3$ to formalin, following which the tissue was stained by the Marchi method, seemed definitely to reduce the amount of dust, and to lighten the background without apparent decrease in the staining of the degenerating myelin. Experiments 25 and 26 show that the addition of 1 and 5% $KClO_3$ to formalin reduced the staining of the background in proportion to the percentage of oxidizing agent added. It was found also that lengthening the time of such fixation to 2 days improved the quality of staining still more. Figures 1 and 2 show the changes produced in tissue from the same spinal cord by the addition of $KClO_3$ to the fixative.

Fine granular deposits of reduced osmic acid resembling dust were present in the interstitial spaces of all control preparations. Usually



EXPLANATION OF PLATES

Figures 1 and 2. Preparations of spinal cord from the same cat (following cortical injury) stained in Marchi's solution plus 2% acetic acid. Preparation shown in Figure 1 was fixed in 10% formalin and that in Figure 2 in 10% formalin plus 1% $KClO_3$.

Figure 3. Spinal cord of cat (normal area) fixed in formalin 10% and monochloracetic acid 1%, followed by staining in Marchi's stain, which shows the staining of normal myelin rings as a result of the acidification of the fixative. × 300.

Figure 4. Spinal cord of cat (normal area) fixed in formalin 10% and stained in Marchi's solution plus 2% acetic acid (× 300), showing an excessive amount of interstitial granular deposits. Several degenerating globules are present in the field.

Figures 5 and 6. Preparations of spinal cord from the same cat (following cortical injury) stained in Marchi's solution plus 2% acetic acid. Preparation in Figure 5 was fixed for 1 day and that in Figure 6 for 1 year in 10% formalin before staining. Note staining of normal myelin sheaths and loss of degenerating myelin in Figure 6.

the deposits were very fine and scarcely noticeable, but at other times coarse black granules, which could be mistaken easily for scattered degeneration products, were found (fig. 4). Its striking characteristic was the variability with which it occurred in different animals (confirming Duncan, 1930).

It is unfortunate that the stainability of this dust is very similar to that of degenerating myelin. In experiments in which the tissues were washed before staining in Marchi's solution (with or without 2% acetic acid) granular deposits were reduced to a minimum, but in so doing shrinkage of the degenerating globules with a resultant loss in their distinctness occurred. It was noticed also that the addition of acetic acid to the Marchi stain or better still the employment of formalin-$KClO_3$ fixation (formalin 10% and $KClO_3$ 1% for 48 hours—Group G) redueed markedly the amount of dust without noticeably changing the degenerating myelin. In a number of earlier experiments in which a chlorate-osmic stain was used the granular deposits could be eliminated, but only at the risk of inhibiting degenerating myelin from staining. Prolonging formalin fixation or increasing the strength of formalin (20%) increased the dust.

EMBEDDING WITH LOW VISCOSITY NITROCELLULOSE

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Abstract.—**The gelation and cutting of embedding masses of low viscosity nitrocellulose (L. V. N.) and of celloidin were compared. L. V. N. forms a firmer mass which can be cut into thinner sections than celloidin. It tolerates considerable water (up to 6%) in a solvent system of alcohol-water-ether, thereby permitting the use of 95% alcohol instead of absolute for making up the solution. The fluidity of its solutions permits transfer of tissue directly from alcohol-ether to a 20% embedding solution. Faults of L. V. N. are: the nitrated cotton lint contains some grit (hence its solution should be allowed to settle), the sections cut from it are somewhat more easily torn than celloidin sections, and it is sufficiently soluble in absolute alcohol to preclude the use of this fluid in handling sections.**

Ruby[2] has reported the use of low viscosity nitrocellulose for embedding histological material. He called attention to the advantages of manipulation and the lower cost of such material compared with celloidin. Since the source of the material was not stated in the report, we communicated with him and learned that it was Hercules Powder Company's *R. S., 0.5 second nitrocellulose.*

We have confirmed his results relative to the successful use of this material and have made a series of experiments to test its tolerance to water in the alcohol-ether mixtures used as a solvent. The object was to determine to what extent 95% alcohol could be substituted for absolute.

Experimental

A. *Making up the solution.* Since the material comes as a cotton lint moistened with absolute alcohol, no washing is necessary as with celloidin. Solution is most easily effected by adding the necessary amount of alcohol to the cotton, breaking up the large lumps, and then adding the requisite amount of ether.

Our solutions of minimum water content were made by weighing rapidly (to prevent excessive evaporation of alcohol) 100 g. of nitrocellulose, adding 100 cc. of absolute alcohol and finally 140 cc. of anhydrous ether. About 70 g. of nitrocellulose was thus con-

[1]Contribution No. 205.

[2]Ruby, S. Anat. Rec., **55**, Suppl., **74** (Abs. No. 166) 1933.

tained in a solution whose final volume was about 320 cc. and whose water content not over 1% (absolute alcohol 1.5%, anhydrous ether 0.5% or less). Solutions to which water was added contained 2, 4, 6, 8 and 10% of extra water in addition to the 1% unavoidably present without the use of special reagents. Control solutions of 12% celloidin without added water were used for comparison with the nitrocellulose solutions.

B. *Evaporation and gelation.* Specimen vials and Stender dishes were used. The former were not very satisfactory and it seems (altho not emphasized in the texts on histological technic) that a large surface area of the evaporating dish in proportion to the depth of solution is one of the requisites for excellence in nitrocellulose embedding. Both dummy blocks and blocks containing tissue (central nervous system) were used. Evaporation was as rapid as possible without permitting the formation of bubbles.

C. *Cutting.* A precision microtome was used and blocks were cut both dry and wet, after hardening in 70 or 80% alcohol. The cutting qualities were judged chiefly by the minimum thinness of section that it was possible to obtain, but the friability of the block and tendency of sections to tear were noted as well.

Results

Complete solution was effected with low viscosity nitrocellulose with even 10% water added, altho this amount was obviously detrimental to the formation of a satisfactory embedding mass. Six percent was taken as the upper limit of tolerance and 2% improved both the rate of gelation and final cutting qualities of the blocks.

Firm blocks of low viscosity nitrocellulose could be obtained with an evaporation time of about half that required by celloidin. Less shrinkage occurred during evaporation and subsequent hardening. These findings confirmed expectations, because the nitrocellulose solution was almost twice as concentrated as the celloidin.

A 22% solution of the low viscosity material was sufficiently fluid to penetrate after direct transfer of tissue from alcohol-ether to it without using intermediate concentrations.

Serial sections as thin as 4μ were obtained from one of the best blocks. Seven micra sections could be cut from any of the blocks made from solutions which did not contain more than 4% added water. Celloidin blocks, cut under the same conditions could not be cut thinner than 12μ satisfactorily. Blocks of L. V. N. could be cut better with a dry knife than a wet one, and it was necessary only to moisten the sides of the blocks occasionally with 70 or 80% alcohol.

Points of difference noted in the manipulation of L. V. N. and celloidin were as follows:

1. Blocks of L. V. N. need not be allowed to become quite as firm thru evaporation (before hardening in alcohol or chloroform) as celloidin.

2. L. V. N. is more soluble in absolute alcohol than celloidin, and difficulty was encountered when sections were dehydrated with absolute alcohol, prior to mounting. The use of butyl alcohol between 95% ethyl alcohol and xylene obviated the trouble. (Since some laboratories use oils for clearing after 95%, the softening would not be encountered.)

3. Sections from blocks of L. V. N. were a little less tough than celloidin sections of the same thickness.

4. L. V. N. tends to stick to glass more than celloidin; but this tendency was corrected by oiling the glass with a thin film of mineral oil.

5. Grit that is present in the cotton lint will dull knives unless solutions are allowed to settle a week or two and decanted, or centrifugalized before use.

Points related to economy in the use of L. V. N. are:

1. The cost of L. V. N. per pound is about the same as celloidin per ounce, and altho it takes twice as much of the former, there is still a large margin of economy.

2. Ninety-five percent alcohol (without added water) may be used in making up the solution. Our experimental results indicate that the material works just as well or better when 95% alcohol is substituted for absolute.

3. Time is saved in making up the solution (only one solution 20 to 25% is needed) and also in getting material embedded.

A BLUE STAIN FOR MICROORGANISMS IN HUMUS AND IN SOIL

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ABSTRACT.—**For better color contrast with humus-colored dead organic matter, a bluish stain was sought for replacing the pinkish dyes in use for staining microörganisms in soil. Among dyes tried, fast acid blue R (C. I. 760) was found to stain selectively enough and optimally in hue. A didymium glass may serve for optical differentiation, if needed.**

The direct microscopy of microorganisms in soil requires staining to differentiate the organisms from dead soil colloids. Stains selective enough for the purpose were found by Conn (1918) and later workers only within the fluorescein group of dyes. These are all red to pink of different hue, having an absorption maximum in green and a second, mostly weaker maximum in blue to deep green. The first maximum is situated in aqueous solution anywhere from 0.51 to 0.55 mμ wavelength (see Conn, 1929; Schultz, 1923; also Colour Index):

Dyes	Reference numbers		First absorption max.
	Schultz	Colour index	μ wavelength
Erythrosin Y	591	772	0.51
The eosins	587–590	768–771	0.52
Erythrosin B	592	773	0.53
The phloxines	593, 594, 596	774, 776, 778	0.53–0.54
Rose bengal	(595), 597	(777), 779	(0.54–) 0.55

The favor has been with the last three hues, but in general not because of the hue, and with a curious disagreement between authors as to the best choice of dye. Thus, Winogradsky (1925, p. 325) blamed Conn's rose bengal, Conn (1926, p. 126) one of Koffman's phloxines, and Koffman (1929, p. 342) Winogradsky's erythrosin (C. I. 773) for not being selective enough. Various observations (Winogradsky, 1925, p. 325; Koffman, 1929, p. 346) and systematic studies by Conn and Holmes (Conn, 1926; Conn and Holmes, 1926 and 1928) suggest accidental solubility effects rather than inherent differences in selectiveness between dyes as an explanation to the preference variously given to any of the deeper pink stains. None of the dyes recommended represents the ideal dye, staining nothing but the soil organisms, and the best contrast is obtained with a suitable middle staining intensity (Conn, 1928; Conn and Holmes, 1928).

Conn alone (1932, p. 234; Conn and Holmes, 1926, p. 93) seems thus far to have considered the importance of the hue as such. The degree of color contrast becomes particularly important in the study of preparations rich in humus. For such study, the writer has long felt dissatisfied even with rose bengal, the deepest pink stain proposed. The color contrast with humus colors is in itself not poor, but the stain does not bring out as much as desired of the bacterial life among the dead organic matter, because the pink and yellow blend to salmon and brick red and similar shades. These resulting colors are often difficult to distinguish against a humus background which is in the stained preparations always redder than its natural color, due to traces of stain.

A search was made for a suitable dye of different absorption characteristics. It was conducted along two lines. Violet to blue acid dyes having the same or nearly the same affinities as the fluorescein dyes to different fibers were picked from the Colour Index, independently of constitution. Irrespective of fiber affinities, bluish acid dyes were picked if constitutionally similar to fluorescein. By no means all of the dyes so chosen could be procured, but a fair number were tried. Many of those were obtained thru the courtesy of Dr. H. J. Conn, of the N. Y. Agr. Exp. Station at Geneva, N. Y. (most of his samples being originally furnished him by the courtesy of the Color and Farm Waste Division of the Department of Agriculture at Washington, D. C.), and of Dr. Aug. Merz, of the Calco Chemical Company, Bound Brook, N. J. To both of these gentlemen, the writer is also much indebted for helpful suggestions.

The dyes were tested with and without phenol and over a pH range from 1.3 to 7 or sometimes 8. Most dyes were ruled out in preliminary macroscopical tests on smears of albumen and of agar suspensions of humus precipitated with either acid or lime. All but two dyes were discarded in tests on soil on account of not being selective enough, mainly due either to hue (see the following) or to their coloring the colloids too generally or plasma too weakly. One dye, closely related to fluorescein (Schultz 600, C. I. 782; laboratory preparation) was found promising but was discarded because of the dull colors of the stain (purple to greenish ink-blue, depending on after-treatment). The dye finally found satisfactory is also related to fluorescein (cf. the findings of Koffman, 1929, p. 342).

This one dye was found far superior to any other tested. It is Schultz 584, C. I. 760. A small sample "violamin 3B, C. I. 760" was obtained thru Dr. Conn from the Color Laboratory in Washington, but practically all tests refer to "fast acid blue R" received from

General Dyestuffs Corporation with the statement: "Manufactured by the I. G[1]. and represents the original MLB[2] type". Both products appear identical as to spectral transmission[3]. In a comparative test, the General Dyestuffs' product seemed to stain better in plain aqueous solution than did the other brand. This difference may or may not be real. The writer has no doubt as to the substantial identity of the two products.

The fast acid blue R was included among the dyes first suggested for trial by Dr. Conn, but it was for some time omitted from the tests largely because its absorption maximum is erroneously located at 0.53μ wavelength both by Shultz (1923) and in the Colour Index (1924). The maximum lies roughly at 0.56μ (estimated) in the aqueous solution which is violet to purplish in high dilution. The dye stains surprisingly bluer, a slightly violet blue, with the absorption maximum at about 0.58μ, as determined spectrophotometrically on stained gelatin film.

Among all dyes tried, including the fluorescein dyes recommended by previous authors, fast acid blue R has given the best color contrast in stained preparations of humus. This is due partly to its affinity to plasma and low affinity to dead humic matter, but very largely to the optimal hue of the stain. Any dye staining a pure blue to sky-blue would again yield a poorer color contrast. It would do so even if perfectly selective, staining nothing but the soil organisms.

The point is best made and explained in the terms of the old Hering or the similar new Houstoun (1932) theory of color vision. The color of any blue stain contains either a red or a green component, or color valency, depending upon the position and shape of the absorption band or bands. "Pure" blue would occur as a boundary case, if the antagonistic red and green valencies balanced each other exactly. Thus the blues can be divided into two classes, viz., reddish and greenish blues. These behave strikingly differently when combined with yellows such as the humus colors.

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[1]I. G. Farbenindustrie, the cartel of German dye manufacturers.

[2]Meister Lucius and Brüning; the old name of the Dye Works at Höchst am Main, Germany.

[3]Thus the "violamin 3B" from the Color Laboratory is not likely to be the mixture mentioned under Schultz 584 and C. I. 760. It is true that the absorption was compared by ocular observation only, using a bright prismatic spectrum, 15 cm. long. Yet violamin 3B, also according to information given by the General Dyestuffs Corporation, is "a mixture based on fast acid blue R". For the fast acid blue R, it was stated: "To the best of our knowledge, there is no other manufacturer" (i.e., no other than the I. G. Farbenindustrie) "and we do not believe that a similar product is available from other sources." For the reasons given, the designation fast acid blue R is used here rather than violamin 3B. The use of the latter name in ordering the dye may lead to confusion.

Humus colored matter absorbs gradually from the blue end of the spectrum. The yellow colors of thinner layers result from the removal of violet to blue, while green to red is still represented, with the green and red components about equally strong. When superimposed upon this yellow, every greenish-blue will yield green, but not so the reddish-blues. With the latter, the green component will never get the upper hand over the red valency, provided the blue stain is reddish enough to make up for the lost red valency of the spectral violet. The resulting combination colors will be different shades of blue, grey and yellow, but not green. The same difference is seen when comparing the transition colors of some of the currently used Clark and Lubs' indicators. The clear blue colors of brom cresol green and brom thymol blue change to yellow thru green, but the slightly violet blue thymol blue, just as well as the more violet brom phenol blue, changes thru indistinct shades of greyish where there is very little or no green.

The difference referred to is very important in staining humus for bringing out the organisms, as shown for instance by a comparison of fast acid blue R with patent blue (Schultz 543, C. I. 712) which was carried along for a considerable time in the tests. The latter, a very brilliant greenish blue, could occasionally be made to give beautiful color contrast between yeast cells and precipitated humus mixed with them, but it completely failed in tests on natural humus. Mere traces of stain make the humus colors distinctly green, and stained cells viewed against humus colored background are green also, resulting in ugly-looking preparations with poor color contrast. The same thing is sure to happen with any pure to greenish blue. Fast acid blue R, on the other hand, gives a good and pleasing color contrast, the stained organisms remaining different shades of blue and the humus colored matter different shades of yellow. Traces of stain make the yellow colors somewhat greyish but do not change them into green.

Since fast acid blue R is a powerful coloring matter, it has to be used in higher dilution than the halogen substituted fluoresceins. Thus far, the most consistently good results have been obtained staining (cold) overnight in staining jars with a 0.05% solution of the dye in 4% phenol.[4] No washing is then needed. An optical

[4]Since the technic of preparing the smears may influence the results, the writer's procedure may be shortly described. It has not been very standardized nor is it very elaborate, the writer's aim being simply to have the material spread out thin enough to permit microscopical examination, at the same time retaining as much as possible of the original arrangement of its elements. The sample is stirred up in 20 or more times its volume of a cold agar solution. About 0.1 ml. of the suspension is spread out

differentiation can be effected, if needed for parts of the preparation, by interposing a Corning didymium glass. This glass has an extremely sharp absorption band in yellow to golden yellow, nearly coinciding with the peak of the absorption curve of the fast acid blue R stain. The blue sensation which would result from this absorption is very nearly balanced by weaker bands in blue, so the glass has little color and changes the color of the illuminating beam only slightly. Its chief effect on the stained preparation is to wash out, as it were, the blue stain where there are only traces of it, without affecting the depth of staining of stronger stained elements.

Unlike the halogen substituted fluoresceins, fast acid blue R is rather fast to light and does not seem to be affected by salts of lime in moderate quantities. This appears as a further advantage. The indifference to lime would seem to obviate the need of adjusting the staining solution individually to different soils, as recommended with the fluorescein dyes (Conn 1928; Conn and Holmes, 1928).

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on a thin cover glass so as to cover 1–2 square centimeters, dried on a luke-warm hotplate, fixed with alcohol and stained. The agar solution is made of such a strength that it does not quite gelatinize at room temperature, or does so only after long standing (for instance, 0.2%; varying with different lots of agar). It is filtered hot thru a Seitz bacterial filter to remove the numerous bacteria which may be found in agar. Sometimes, 0.01% of thymol was added as a further precaution to prevent new growth in broken lots of solution. The stained smears were examined in plain water, or phenolated water in the case of sealed preparations.

METHODS FOR THE STANDARDIZATION OF BIOLOGICAL STAINS

PART V[1]

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MISCELLANEOUS DYES

Abstract.—**In this paper are given the methods for determining the suitability of certain dyes of the pyronin, thiazin, oxazin, azin and natural dye groups for certification by the Commission on Standardization of Biological Stains. These methods have been developed by the Commission in coöperation with the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture. The dyes for which the methods are given in the present paper are: Pyronin G, pyronin B, neutral red, safranin, nigrosin water-soluble, brilliant cresyl blue, cresyl violet, Nile blue A, thionin, methylene blue, methylene azure (azure A), azure C, toluidine blue O, indigo carmin (indigotine) and carmin. For each of these dyes methods are discussed under the following headings: (1) identification or qualitative examination; (2) quantitative analysis; and (3) biological tests.**

A general discussion of the equipment and technic employed in the identification and quantitative examination of biological stains has been given in previous papers of this series, Part I and Part III.[1] The formula used for the calculation of dye content of samples analyzed by means of reduction with titanous chloride is as follows:

$$\frac{\text{cc. } TiCl_3 \text{ used} \times \text{normality factor} \times \text{molecular weight of} \times \text{dye } 100}{\text{weight of sample} \times \text{no. of hydrogen equiqalents} \times 1000} = \text{Per cent dye;}$$

or

$$\frac{\text{cc. } TiCl_3 \text{ used} \times \text{normality factor} \times 10 \times 100}{\text{cc. of N/10 } TiCl_3 \text{ required per gram of dye} \times \text{weight of sample}} = \text{Per cent dye}$$

[1]The previous papers of this series have appeared in Stain Techn., **8**, 87, 95, 121 (1933); **9**, 41 (1934).

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PYRONIN G, C. I. NO. 739[4]

Identification: Pyronin G is tetramethyldiaminoxanthenyl chloride, $C_{17}H_{19}N_2OCl$. The absorption ratio (E at 530 mμ/E at 560mμ) is 0.80. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Since chemical methods of analysis are unsatisfactory for this dye, it is analyzed by means of the spectrophotometer.[5] Dissolve 1 g. of dye in a liter of distilled water. Dilute a 5 cc. aliquot of this solution to 250 cc. with water and then further dilute a suitable portion of this second solution with an equal volume of 95% alcohol. Measure the extinction coefficient, (E), of the alcohol-water solution in a 1 cm. layer at 550 mμ. The percentage of anhydrous dye in the original sample is calculated by means of the following formula:

$$\% \text{ dye} = \frac{E \times 100}{3.15}$$

Biological Tests: Pyronin is tested in staining gonorrhoeal smears by the Pappenheim-Saathof stain, as given on page 119 of Biological Stains, 2nd edition. With a good sample, the cocci should be bright red and the nuclei green (not purplish).

PYRONIN B, C. I. NO. 741

Identification: Pyronin B is tetraethyldiaminoxanthenyl chloride, $C_{21}H_{27}N_2OCl$. The absorption ratio (E at 530 mμ/E at 560 mμ) is 0.55. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Prepare a solution of dye in a mixture of equal parts of distilled water and 95% alcohol exactly as described in the method for pyronin G. Measure the absorption (E) of this solution at 555 mμ in a 1 cm. layer. The percentage of anhydrous dye in the original sample is calculated by means of the following formula:

$$\% \text{ dye} = \frac{E \times 100}{3.68}$$

Biological Tests: Pyronin B is tested by the same procedure as pyronin G.

NEUTRAL RED, C. I. NO. 825

Identification: Neutral red is aminodimethylaminotoluphenazonium chloride, $C_{15}H_{17}N_4Cl$. The absorption ratio (E at 515 mμ/E at 545 mμ) is 0.75. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water which contains 5 drops of glacial acetic acid per 50 cc. of solvent.

Method of Analysis: Neutral red is analyzed by means of the spectrophotometer, using a method similar to that given for the pyronins.[5] Dissolve 1 g. of dye in 1 liter of distilled water. To 5 cc. of this solution add 5 cc. of glacial acetic acid and dilute to to 250 cc. with distilled water. Then dilute a suitable portion of the second solution with an equal volume of 95% alcohol. Measure the extinction coefficient of this solution in a 1 cm. layer at 540 mμ. The percentage of anhydrous dye in the original sample is calculated by means of the following formula:

$$\% \text{ dye} = \frac{E \times 100}{1.68}$$

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[4]This is the number under which the dye is listed in the Colour Index, published by the Society of Dyers and Colourists, Bradford, Yorkshire, 1924.

[5]Holmes, W. C., and Peterson, A. R. The analysis of neutral red and of the pyronins, Stain Techn., 5, 91. 1930.

Biological Tests: Neutral red is tested for two purposes: vital and supravital staining of protozoa; differential staining of living cells of blood and connective tissues.

In testing for the first purpose, not only color intensity but relative toxicity and the stability of aqueous solutions in sunlight are determined. *Paramecium* and erythrocytes of *Necturus* are utilized as test materials. With *Paramecium* aqueous solutions ranging from 1:5000 to 1:100,000 concentration are employed, and one drop of conconcentrated culture is added to 10 cc. of the dye solution. The rate and intensity of staining of intracellular granules, the effect on ciliary activity and elevation of the pellicle, and the distortion of the organism constitute the criteria of the suitability of the dye for vital staining. In supravital testing a 1:1250 solution in absolute ethyl alcohol is applied to a glass slide, yielding the so-called dry-dye-film. Drops of freshly drawn blood are placed on the slide, covered, and sealed with warm vaseline. Observations are made at room temperature with the preparation shielded from direct heat from the source of illumination. In the erythrocyte, definite bipolar clusters of preformed granules stain immediately. Relative toxicity is judged by the rate at which induced or secondary staining patterns (granules or reticulation) appear. Corrections for percentage of dye content and the kind of salt (chloride or iodide) are necessary.

In testing for the second purpose (staining living blood and connective tissue cells), a saturated solution of the dye (125 mg. to 50 cc.) is made in neutral absolute alcohol, freshly distilled from lime. A dilute solution is made by adding from 30 to 50 drops of the saturated solution to 10 cc. of neutral absolute alcohol. From the dilute solution, scrupulously cleaned slides are prepared with a dry film of the dye by flooding them with the solution, draining it off quickly and drying them in an upright position in a current of warmed air. The biological testing is made with blood cells; rabbit's blood is convenient on account of the high percentage of basophilic leucocytes and the adequate number of eosinophiles. The film of blood should be sealed quickly with a vaseline of high melting point, such as Salvolene, and the preparations studied in a warm box at 37° C. The test of the dye involves both the color of the granules of the granulocytes and the motility of the cells. The basophilic granules should stain a brilliant red, corresponding to the color of neutral red in acid solution; the eosinophilic granules should be yellow, or slightly orange, corresponding to the dye in alkaline solution; the neutrophilic granules should be either the color of the neutral solution of the dye or show almost no stain. The vacuoles of the monocytes also show the neutral tone of the dye. In motility, the neutrophilic leucocytes should be in constant amoeboid motion for at least an hour and the eosinophilic leucocytes for half an hour.

SAFRANIN, C. I. NO. 841

Identification: Safranin is a mixture of diaminophenylditolazonium chloride, $C_{20}H_{19}N_4Cl$ and diamino-o-tolylditolazonium chloride, $C_{21}H_{21}N_4Cl$. The absorption ratio of the dye (E at 515 mμ/E at 545 mμ) is 1.02. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in a mixture of 75 cc. of distilled water and 75 cc. of 95% alcohol, heat to boiling. add 30 cc. of 30% sodium tartrate solution, and titrate with N/10 $TiCl_3$ solution to a pale yellow end point. Some samples give a sharper end point when titrated in the cold. The following data are used for determining the percentage of anhydrous dye in the original sample:

Molecular weight, $C_{20}H_{19}N_4Cl$ 350.641
Molecular weight, $C_{21}H_{21}N_4Cl$ 364.657
Hydrogen equivalents per mol of dye ..... 2.
cc. of N/10 $TiCl_3$ per gram of dye (average of two homologs) 55.942

Biological Tests: Safranin is tested as a chromosome stain in the Flemming triple stain and in combination with light green SF yellowish or fast green FCF. The procedure for the Flemming stain is the same as described in the last article of this series under crystal violet (Stain Techn., **9**, bottom p. 46). The procedure with fast green or light green as a stain is given elsewhere in the same article (bottom p. 43).

NIGROSIN WATER-SOLUBLE, C. I. NO. 865

Identification: Nigrosin water-soluble is the sodium salt of the product resulting from the sufonation of spirit-soluble nigrosin which is obtained by the interaction of anilin, anilin hydrochloride and nitrophenol (or nitrobenzene and iron). The absorption ratio of this dye (E at 575 mμ/E at 605 mμ) is 1.01. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Since this dye is a variable mixture of complex compounds of unknown constitution, quantitative determination of the dye content is impossible.

Biological Tests: Nigrosin is tested in Dorner's spore stain by the first procedure given on page 85 of Biological Stains, 2nd edition. For this test a 2 to 4 day old culture of some rapid spore-former such as *Bacillus cereus*, is used.

BRILLIANT CRESYL BLUE, C. I. NO. 877

Identification: In general, samples of this stain are aminodimethylaminomethyldiphenazonium chlorides, $C_{15}H_{16}N_3OCl$, altho the dye is given in the Colour Index as the diethyl derivative. The absorption ratio (E at 605 mμ/E at 635 mμ) is 0.90. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of the dye in a mixture of 150 cc. of 95% alcohol and 100 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution. The end point is brown and often difficult to detect. In order to prevent or lessen precipitation in the reduced solution, the sodium acid tartrate may be dissolved in an additional 50 cc. of hot distilled water and added after the dye solution has been heated to the boiling point. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight 289.609
Hydrogen equivalents per mol of dye ..... 2.
cc. of N/10 $TiCl_3$ per gram of dye 69.060

Biological Tests: In testing brilliant cresyl blue, fresh blood is examined under a cover glass on which a filtered 0.3% alcoholic solution of brilliant cresyl blue has been dried or blood films are made on similar cover glasses, dried and counterstained with Wright's or similar stain. The reticulum of immature red cells should be a clear-cut blue on a very pale blue (fresh) or eosin colored (stained) background. Blood platelets should stain a pale blue and be discrete, with only a minimum of precipitate or debris.

CRESYL VIOLET[6]

Identification: The American stain cresyl violet (National Aniline Company) is aminonaphthodimethylaminotolazoxonium chloride, $C_{19}H_{18}N_3OCl$. The absorption ratio of this dye (E at 620 mμ/E at 650 mμ) is 1.16. The solvent used is a mixture of

[6]This dye has no Colour Index number

equal volumes of 95% alcohol and distilled water. On the other hand, cresyl echt violet (Grübler) is a dye of unknown constitution, which has an absorption ratio of 7.75 (E at 620 mμ/E at 650 mμ) using the same solvent as above.

Method of Analysis: Dissolve 0.3 g. of dye in 200 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling, and titrate with N/10 $TiCl_3$ solution to a greenish yellow end point. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	339.625
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	58.880

Biological Tests: Cresyl violet is tested as a stain for fresh tissues, especially those removed from tumors at biopsy. As very few samples of this dye have as yet been submitted for certification, no exact biological tests have been worked out.

NILE BLUE A, C. I. NO. 913

Identification: Nile blue A is aminodiethylaminonaphthophenazonium sulfate $(C_{20}H_{20}N_3O)_2SO_4$. The absorption ratio of this dye (E at 620 mμ/E at 650 mμ) is approximately 0.90.[7] The solvent used is a mixture of equal parts of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of dye in a mixture of 40 cc. of distilled water and 120 cc. of 95% alcohol, heat to boiling, add 30 cc. of 30% sodium tartrate solution, and titrate slowly with N/10 $TiCl_3$ solution. It is necessary to keep the solution boiling thruout the reduction, otherwise the reaction is very slow, especially near the end point. The final color change is from reddish brown to yellow. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	732.432
Hydrogen equivalents per mol of dye	4.
cc. of N/10 $TiCl_3$ per gram of dye	54.612

Biological Tests: Nile blue sulfate is tested as a differential fat stain by the Smith and Mair technic. The material used for this test consists of insect ova fixed and preserved in neutral formalin. Sections are cut on the freezing microtome and stained with the hydrolyzed dye prepared by the technic given on page 75 of Biological Stains, 2nd edition. After staining for about 2 hours at 37.5° C. they are differentiated in 2% acetic acid, washed in water and mounted in glycerin or Farrant's medium. A good sample is indicated by its ability to distinguish between free fatty acids (blue) and neutralized fats (red).

THIONIN (LAUTH'S VIOLET), C. I. NO. 920

Identification: Thionin is diaminodiphenazthionium chloride, $C_{12}H_{10}N_3SCl$. The absorption ratio of this dye (E at 590 mμ/E at 620 mμ) is 1.44. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 200 cc. of distilled water, add 15 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution to a pale yellow end point. The dye mixture must be kept hot during the titration to prevent precipitation. The following data are used in calculating the percentage of anhydrous dye in the original sample:

[7] As available samples of this stain are of a somewhat diverse character, it may eventually prove advisable to revise the value given for the absorption ratio.

Molecular weight	263.625
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	75.865

Biological Tests: Thionin is tested as a stain for frozen sections, also in Stoughton's method for sections of plant pathological material. For frozen sections the dye is prepared in a 0.5% solution in 20% ethyl alcohol. The sections, immediately after cutting, are immersed in water and placed briefly (1 to 2 minutes) in the stain, then washed and mounted in water for examination. Good differentiation of the nuclei is regarded as the criterion of a satisfactory sample. In the Stoughton method, plant pathologic material is fixed in one of the usual botanical fixatives, paraffin sections are stained for 1 hour in 0.1% thionin in 5% aqueous phenol, dehydrated in successively stronger alcohols and differentiated in a saturated solution of orange G in absolute alcohol. They are then washed in absolute alcohol, cleared in xylol and mounted in balsam. Fungal parasites should be violet to purple; cell walls yellowish or green; lignified tissue, blue; host nuclei, blue with purple nucleoli; chromosomes, deep blue; and nuclei of the fungal hyphae or spores, deep purple.

METHYLENE BLUE, C. I. NO. 922

Identification: Methylene blue is tetramethyldiaminodiphenazthionium chloride, (tetramethyl thionin) $C_{16}H_{18}N_3SCl$. This dye is sometimes marketed for textile purposes as the zinc chloride double salt, but the dye in this form is not recommended for use as a biological stain. The absorption ratio of the dye (E at 635 mμ/E at 665 mμ) is 0.60. The solvent used is a mixture of equal parts of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 150 cc. of hot distilled water. (Care must be taken to attain complete solution.) Add 10 g. of sodium acid tartrate, heat to boiling, and titrate with N/10 $TiCl_3$ solution. The end point is pale yellow and quite sharp. If any undissolved particles remain in the bottom of the flask, they will show up at this point, slowly coloring the solution. Coloration due to undissolved material must not be confused, however, with the return of color due to the oxidizing action of the air. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	319.689
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	62.560

Biological Tests: Methylene blue is tested for histological and bacteriological staining and as a constituent of Wright's blood stain. As an histological stain it is tested on paraffin sections of tissue fixed in Zenker's fluid, with phloxine as a counterstain, by the procedure given at the bottom of page 131, Biological Stains, 2nd edition. A good sample should show good nuclear staining without removing the phloxine from the cytoplasm.

As a bacteriological stain it is tested for staining the diphtheria organism and for staining bacteria in milk. For the former purpose smears from a throat culture of a case of diphtheria are stained in three different solutions of the sample under examination; namely Loeffler's formula, alcoholic aqueous methylene blue (both given on page 67 of Biological Stains, 2nd edition), and also in a 1% aqueous solution of the dye. The solutions are applied to the preparation for a few seconds and are then examined under the microscope to see if the typical barred or granular structure shows. A good sample should show this typical staining with all three solutions.

For staining bacteria in milk, the method given on page 69 of the above-mentioned book is followed. A good sample should not remove the milk smear from the slide and should show deeply stained bacteria in a light blue background.

In testing as a constituent of Wright's stain, the same procedure is followed as in the case of eosin.

METHYLENE AZURE, C. I. NO. 923

Identification: Methylene azure is obtained by the oxidation of methylene blue, which results in a mixture of di-, tri- and tetramethyldiaminodiphenazthionium chlorides in varying proportions. The first-mentioned product, asymmetrical dimethyl thionin or azure A, $C_{14}H_{14}N_3SCl$, which is more often found to be the main constituent of samples of this dye, has an absorption ratio (E at 635 mμ/E at 665 mμ) of 2.24 when a solvent of equal mixtures of 95% alcohol and distilled water is employed. Azure B or trimethyl thionin, $C_{15}H_{16}N_3SCl$, and has an absorption ratio (E at 635 mμ/E at 665 mμ) of 1.34. This homolog of thionin has no particular merit as a stain, but its detection in samples of methylene azure is necessary for a complete analysis of the dye. The ratio for methylene blue has previously been given. From these three ratios it is possible to estimate with fair accuracy the chemical nature of samples of this stain.

Method of Analysis: Dissolve 0.3 g. of dye in 150 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution to a yellow end point. If qualitative examination has shown the dye sample to be almost pure azure A or almost pure azure B, the percentage of anhydrous dye in the original sample may be determined from the following data. If the dye is found to be a mixture, the actual dye content cannot be accurately determined.

	Azure A	*Azure B*
Molecular weight	291.657	305.673
Hydrogen equivalents per mol of dye	2.	2.
cc. of N/10 $TiCl_3$ per gram of dye	68.574	65.428

Biological Tests: Azure A is tested on paraffin sections of animal tissue fixed in Zenker's fluid by means of Haynes' modification of French's stain, as given on page 63 of Biological Stains, 2nd edition. Good nuclear staining is regarded as the criterion of a satisfactory sample.

AZURE C[8]

Identification: Azure C is monomethyldiaminodiphenazthionium chloride (monomethyl thionin), $C_{13}H_{12}N_3SCl$. The absorption ratio of this dye (E at 605mμ/E at 635 mμ) is 1.06. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 150 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	277.641
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	72.032

Biological Tests: Azure C is tested in the same way as azure A (see methylene azure).

[8]This dye is not listed in the Colour Index.

TOLUIDINE BLUE O, C. I. No. 925

Identification: Toluidine blue O is usually the zinc chloride double salt of aminodimethylaminotoluphenazthionium chloride, $C_{15}H_{16}N_3SCl + ZnCl_2$, but may also be prepared as the chloride. The absorption ratio of this dye (E at 620 mμ/E at 650 mμ) is 1.05. The solvent used is a mixture of equal parts of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.3 g. of dye in 150 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution to a yellow end point. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	305.673
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	65.429

Biological Tests: Toluidine blue O is tested as a stain for frozen sections and as a substitute for azure A in Haynes' modification of French's stain. For the former purpose the same procedure is used as in the case of thionin; for the latter the same as in the case of azure A.

It is also tested as a constituent of Albert's diphtheria stain, using the formula and method given on page 71 of Biological Stains, 2nd edition.

INDIGO CARMIN (INDIGOTINE), C. I. No. 1180

Identification: Indigo carmin is the disodium salt of indigotin-5, 5′-disulfonic acid, $C_{16}H_8N_2O_8S_2Na_2$. The absorption of this dye (E at 590 mμ/E at 620 mμ) is 0.84. The solvent employed is 80% alcohol. Monosulfonated indigo, the principal impurity found in indigo carmin, may be readily detected in samples of this latter dye, as the ratio value obtained under the same conditions as above is 1.34 for the monosulfonate, in comparison with 0.84 for the disulfonate.

Method of Analysis: Dissolve 0.5 g. of dye in 200 cc. of distilled water, add 15 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$ solution. The color change at the end point is from blue to reddish brown. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	466.202
Hydrogen equivalents per mol of dye	2.
cc. of N/10 $TiCl_3$ per gram of dye	42.900

Biological Tests: Indigo carmin is tested in Shumway's modification of the Cajal stain. Young amphibian larvae fixed in Bouin's picro-formol-acetic are embedded in paraffin and sectioned. Sections are stained 20 minutes in basic fuchsin (sat. aq. sol.) after which they are rinsed in distilled water and placed in an equal mixture of the indigo carmin to be tested and picric acid (each in sat. aq. sol.) for 5 minutes. They are passed rapidly thru 70% alcohol (when the sections appear red), 95% and absolute alcohol (until the sections appear green) and cleared in xylol. With a satisfactory sample muscle tissue is green and connective tissue blue, contrasting sharply with the red of the nuclei.

CARMIN, C. I. No. 1239

Identification: Carmin is a cochineal product, usually furnished as the aluminum-calcium salt of carminic acid. The exact structure for carminic acid is somewhat uncertain; the molecular formula is $C_{22}H_{20}O_{13}$. It is a strong dibasic acid with a side

chain which contains four hydroxyl groups and possibly possesses a sugar-like structure, altho carminic acid is not a glucoside. It contains at least one asymmetric carbon.

Method of Analysis: Due to the uncertainty of its constitution, no dye content is determined for samples of this stain.

Biological Tests: In testing carmin, Mayer's alcoholic hydrochloric acid stain is prepared by dissolving 4 g. carmin by boiling in 15 cc. distilled water with 30 drops of concentrated hydrochloric acid, then adding 95 cc. of 85% alcohol and neutralizing by adding ammonia until the carmin begins to precipitate. This is employed for staining chick or pig embryos fixed in Bouin's or Zenker's fluid. For sections, the embryos are stained in bulk 24 to 72 hours, without decolorization, embedded in paraffin, and sections are cut 10 to 15μ thick. For toto mounts of both chick and pig embryos, the stain is applied for about 24 hours to secure deep staining, decolorized until rose-pink in 1% HCl in 80% ethyl alcohol, dehydrated and cleared. Small embryos are mounted in balsam and larger ones in benzyl benzoate. A good sample of carmin is indicated by the degree of nuclear staining secured.

ERRATUM

In Stain Technology **8**, 99, a mistake was made in stating the absorption ratio for phloxine B. The parenthetical phrase at the top of the page should read (E at 530 mμ/E at 560 mμ), not (E at 515 mμ/E at 545 mμ).

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